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Exploitation of Gene(s) Involved in 2,4-Diacetylphloroglucinol Biosynthesis To Confer a New Biocontrol Capability to a *Pseudomonas* Strain

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Tn*5* mutagenesis and complementation analysis were used to clone a 6-kb genomic fragment required for biosynthesis of 2,4-diacetylphloroglucinol (Phl) from fluorescent *Pseudomonas* sp. strain F113. A recombinant plasmid, pCU203, containing this region partially complemented a Phl production-negative mutant (F113G22) derived from strain F113. When sugar beet seeds were sown into an unsterilized soil, in which sugar beet was subject to damping-off by *Pythium ultimum*, the emergence of sugar beet seeds inoculated with strain F113 was significantly greater than that of seeds inoculated with F113G22. Transfer of pCU203 into eight other *Pseudomonas* strains conferred the ability to synthesize Phl in only one of these strains, *Pseudomonas* sp. strain M114. Strain M114(pCU203) showed enhanced antagonism towards *P. ultimum* in vitro and significantly increased the emergence of sugar beet seeds in the same soil compared with emergence induced by the parent strain M114.

Evidence for the role of extracellular antibiotics produced by *Pseudomonas* strains in the biocontrol of soilborne plant pathogens has come in part from studies with transposon-induced mutants that are defective in antibiotic production. Thomashow and Weller (20) reported that a Tn*5* mutant of *Pseudomonas fluorescens* 2-79, deficient in the production of phenazine-1-carboxylic acid, was less suppressive to take-all (*Gaeumannomyces graminis* var. *tritici*) than the parental strain. Keel et al. (12) showed that a Tn*5* mutant of *P. fluorescens* CHA0, defective in the production of 2,4-diacetylphloroglucinol (Phl), exhibited reduced suppression to black root rot on tobacco (*Thielaviopsis basicola*) in a gnotobiotic system. Gutterson (7) reported that mutations in the *afuE* locus required for the synthesis of the antibiotic oomycin A by *Pseudomonas* sp. strain HV37A reduced its disease suppressiveness towards *Pythium ultimum*.

The identification of extracellular secondary metabolites involved in biocontrol offers the potential for cloning genes that encode their biosynthesis and using these genes to improve a given biocontrol agent. Haas et al. (9) isolated a cosmid (containing a 22-kb insert) from *P. fluorescens* CHA0 which contained genes involved in the biosynthesis of pyoluteorin and Phl. Increasing the copy number of this fragment in strain CHA0 enhanced the production of pyoluteorin fourfold and that of Phl twofold on agar medium and improved the ability of strain CHA0 to protect cucumber from *P. ultimum* in a gnotobiotic system (9). Bull et al. (2) suggested that it may be possible to design superior biocontrol strains by transferring cloned antibiotic genes into other *Pseudomonas* strains. To this end, they showed that the transfer of a 12-kb fragment from *P. fluorescens* 2-79 into strains of *P. putida* and *P. fluorescens* enabled them to produce phenazine-1-carboxylic acid. Gill and Warren (6) transferred the ability to produce an iron-antagonized fungistatic agent (FAI) from *Pseudomonas* sp. strain NZ130 to *Pseudomonas* sp. strain MK280, increasing the ability of strain MK280 to inhibit the growth of *P. ultimum* on agar medium. A comparison of the ability of these modified and the corresponding wild-type *Pseudomonas* strains to act as biocontrol agents in soil has not been reported. Vincent et al. (22) isolated a cosmid from *P. aureofaciens* Q2-87 which contained gene(s) involved in the biosynthesis of Phl. Mobilization of this cosmid into two heterologous *Pseudomonas* strains conferred the ability to synthesize Phl and increased their activity against *G. graminis* var. *tritici*, *P. ultimum*, and *Rhizoctonia solani* in vitro. In a vermiculite-soil mix, there was no significant increase in the suppression of take-all of wheat or damping-off of cotton by the recombinant derivatives compared with their respective wild-type strains.

One goal of this study was to determine whether Phl production was involved in the ability of fluorescent *Pseudomonas* sp. strain F113 to act as a biocontrol agent on sugar beet seeds in a nonsterilized soil. Gene(s) involved in the biosynthesis of Phl by *Pseudomonas* sp. strain F113 were identified and were also assessed for their ability to confer Phl production in other *Pseudomonas* strains. It was demonstrated that these gene(s) can be used to significantly increase the ability of a *Pseudomonas* strain to increase the emergence of sugar beet seeds, subject to damping-off, in a soil infested by *P. ultimum*.

MATERIALS AND METHODS

Organisms and culture conditions. Wild-type *Pseudomonas* strains used in this study were isolated from the rhizosphere of mature sugar beet, sown at various locations in Counties Cork and Tipperary, Ireland. *Pseudomonas* sp. strains F113 and F113G22 (a Phl-negative mutant of F113, constructed by using Tn*5*::*lacZY*) have been described previously by Shanahan et al. (18). *Pseudomonas* strains F113.T1, F113.T2, and F113.T3 are derivatives of F113, constructed by using Tn*5*::*lacZY*. These three strains were chosen on the basis that they maintained a kanamycin (50 μg/ml)-resistant phenotype during six successive subculturing transfers in Luria-Bertani (LB) broth and did not differ

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from the parent strain in their growth rates in LB or sucrose-asparagine (SA) broth or in their ability to produce Phl on SA agar medium containing 100 μM $FeCl_3$ [SA (+$FeCl_3$) agar]. *Pseudomonas* sp. strain M114 and a spontaneous rifampin (100 μg/ml)-resistant mutant of M114, which was used in this study, have been previously described by O'Sullivan et al. (17).

All *Pseudomonas* strains were maintained on LB agar medium (15) and cultured in SA broth (14) for 18 h at 28°C. *Pseudomonas* strains containing Tn*5*::*lacZY* were cultured in SA broth containing kanamycin (50 μg/ml), while *Pseudomonas* strains containing derivatives of pSUP106 (19) were cultured in SA broth containing chloramphenicol (200 μg/ml). *Pseudomonas* strains used for inoculation of sugar beet seeds (cv. Rex) were first washed three times in quarter-strength Ringer's (Oxoid) solution. *Bacillus subtilis* A1 was maintained on LB agar medium, cultured in LB broth, and washed three times in quarter-strength Ringer's solution prior to use.

Detection of Phl. *Pseudomonas* strains were assessed for their ability to produce Phl by inoculating 100 μl of a bacterial suspension (containing ca. 5×10^8 cells per ml) onto 20 ml of semisolid SA (+$FeCl_3$) agar medium. These agar plates were incubated at 14°C for 4 days. Approximately 6 ml of supernatant, derived after centrifugation of the agar suspension, was then analyzed by the method of Shanahan et al. (18). Briefly, this involved filter sterilizing the supernatant obtained from the agar plates, passing it through a Sep-Pak C_{18} cartridge (allowing the Phl to bind to the C_{18} resin), washing with water, and eluting the antibiotic with methanol. The eluate was taken to dryness, dissolved in 5 ml of mobile phase, and added to a C_{18} column on a Beckman (System Gold) high-performance liquid chromatography (HPLC) system. Elution of Phl was monitored by absorption, using a wavelength of 254 nm and a flow rate of 1.0 ml/min. The concentration of Phl in each agar supernatant was calculated from a standard curve of Phl concentration versus peak area of the HPLC chromatogram, using a pure Phl standard.

Isolation of gene(s) involved in Phl biosynthesis. A plasmid library of strain F113 was constructed by using the IncQ broad-host-range vector pSUP106 (19). This plasmid carries a gene encoding chloramphenicol resistance. In brief, total bacterial DNA of F113 was partially digested with restriction endonuclease *Bam*HI and ligated with pSUP106 which was previously digested with *Bam*HI and treated with alkaline phosphatase. The *Bam*HI restriction site in pSUP106 is directly downstream of the tetracycline resistance gene promoter, and cloning of fragments into this site causes insertional inactivation of the tetracycline resistance gene. This was used as a primary screen for inserts. The ligation mixture was electroporated directly into F113G22, by the method of Farinha and Kropinski (5). The efficiency of electroporation was 10^5 transformants per μg of DNA. Transformants were selected on LB agar medium containing kanamycin (50 μg/ml) and chloramphenicol (200 μg/ml).

To detect transformants of F113G22 containing genomic sequences capable of complementing its deficiency in Phl biosynthesis, colonies were overlaid on SA (+$FeCl_3$) agar by a freshly grown culture of *B. subtilis* A1. *Pseudomonas* sp. strain F113, unlike F113G22, shows pronounced inhibition towards *B. subtilis* A1 on this agar. Transformants of F113G22 which exhibited pronounced inhibition were selected. These transformants were assessed for their ability to produce Phl.

DNA-DNA hybridization procedure. Total genomic DNA was prepared by cesium chloride-ethidium bromide density gradient centrifugation (3, 15). DNA was restricted and immobilized on nitrocellulose by Southern blotting (15). Probe DNA was isolated from a gel by using a QIAEX gel extraction kit (QIAGEN). Hybridization experiments were performed with an ECL Direct Nucleic Acid Labelling and Detection System (Amersham International plc). Conditions specified by the manufacturer were employed in DNA isolation and hybridization experiments.

Plasmid transfer. Plasmids were transferred between *Escherichia coli* and *Pseudomonas* spp. by standard conjugation procedures. These procedures were previously described by O'Sullivan et al. (16).

Plasmid isolation. Plasmid DNA was isolated by the method of Birnboim and Doly (1).

Biocontrol ability of *Pseudomonas* strains in soil. Experiments were conducted in a sandy loam soil (pH 6.9) obtained from Ovens, County Cork, Ireland in which sugar beet had previously been grown. *P. ultimum* was isolated from all seedlings exhibiting the symptoms of damping-off in this soil. Secondary infections were caused by *Fusarium* spp. and *Aphanomyces* spp. The degree of soil infestation was assayed according to methods specified by Jeffers et al. (11) and was recorded as 88 propagules per g of soil. Soil was collected from the upper 5 cm of the soil profile and sieved through a 0.5-cm-mesh screen prior to use. Seeds of sugar beet (cv. Rex) were dipped in washed suspensions (ca. 5×10^8 cells ml^{-1}) of a given *Pseudomonas* strain. Seeds were shown at a depth of 1.5 cm in soil contained in 7.5-cm-diameter pots. Nine seeds were sown per pot, with nine replications per treatment. For controls, seeds were soaked in quarter-strength Ringer's solution or the fungicides Previcur N (Schering AG, Germany) at 20 ml kg^{-1} of seeds and Thiram at 7.5 g kg^{-1} of seeds. Soil moisture was adjusted to 70% of the soil water holding capacity with tap water. Pots were placed in a randomized complete block. Soil moisture was measured every three days and adjusted to 70% of the water holding capacity. Plants were grown for a period of 20 days, after which seed emergence was evaluated.

Bacterial survival on sugar beet in soil. At specified times, three pots from each treatment were selected at random. The seeds or seedlings from each pot were suspended in 9 ml of Ringer's solution and vortexed for 7 min, and a serial dilution was made. Derivatives of strain F113, containing Tn*5*::*lacZY*, were plated on LB medium containing kanamycin (50 μg/ml) and ampicillin (50 μg/ml). Derivatives of strain M114 were plated onto LB agar containing rifampin (100 μg/ml). Control experiments were performed with uninoculated roots.

RESULTS

Role of Phl in increasing seedling emergence. To evaluate the role of Phl, the emergence of seeds inoculated with strain F113 was compared to the emergence of seeds inoculated with a Tn*5*::*lacZY* mutant (F113G22) derived from F113, defective in the production of Phl. The emergence of seeds inoculated with strain F113 was significantly greater ($P < 0.05$) than the emergence of seeds inoculated with strain F113G22 or untreated seeds and equivalent to that of seeds treated with the fungicides Thiram and Previcur N (Fig. 1). This result suggested that the ability to produce Phl is a key component influencing the ability of *Pseudomonas* sp. strain F113 to increase seedling emergence in soil. The emergence of seeds inoculated with F113 was similar to that of a derivative of F113, genetically marked with Tn*5*::*lacZY*

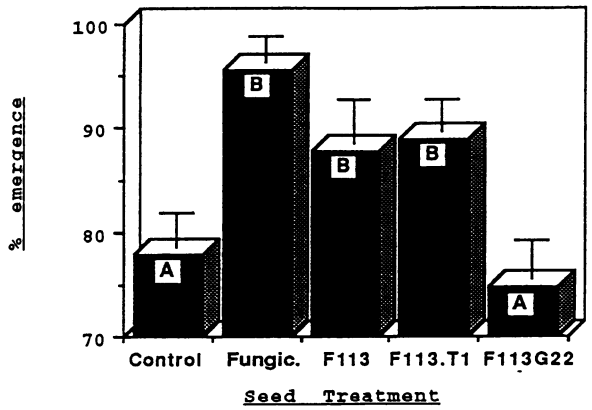

FIG. 1. Emergence of sugar beet seeds inoculated with *Pseudomonas* sp. strains F113, F113.T1, and F113G22 (Phl negative) and control (untreated) and fungicide-treated seeds in a soil subject to damping-off by *P. ultimum*. Columns containing the same letter were not significantly different at the $P < 0.05$ level by analysis of variance. Bars represent standard errors.

(F113.T1). This result suggested that Tn*5*::*lacZY* alone had no influence upon the ability of F113 to increase seedling emergence in soil.

To determine whether the difference in emergence of seeds treated with F113 and F113G22 may have been influenced by a difference in their ability to colonize sugar beet roots in soil, the survival of F113 and F113G22 was monitored in soil. Three derivatives of strain F113 were constructed with Tn*5*::*lacZY* as a marker. Their characteristics are described in Materials and Methods.

During the time course of the experiment, the rates of colonization of sugar beet roots by F113G22 and F113.T1 were not significantly different ($P > 0.05$) (Fig. 2). The rates of colonization of F113.T2 and F113.T3 were also not significantly different ($P > 0.05$) from those of F113.T1 and F113G22. Ten random colonies of F113G22 isolated at each sampling time from the sugar beet rhizosphere exhibited a marked reduction in their ability to inhibit *B. subtilis* A1 in comparison to F113 on SA($FeCl_3$) agar. Ten colonies of F113G22 isolated at the final time of sampling and unable to inhibit *B. subtilis* A1 were unable to produce Phl. It was

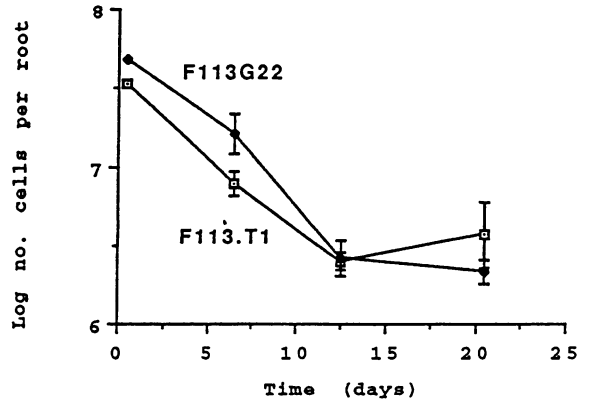

FIG. 2. Colonization of the sugar beet rhizosphere by *Pseudomonas* sp. strains F113.T1 and F113G22 (Phl negative). Bars represent standard errors.

TABLE 1. Production of 2,4-diacetylphloroglucinol (Phl) by *Pseudomonas* strains into which the recombinant plasmid pCU203 was transferred by conjugation

Pseudomonas strain	Phenotype	Phl production (μM)[a]
F113	Phl^+	0.46 ± 0.028
F113G22	Phl^- (Phl::Tn*5lac*)	<0.001
F113G22(pCU203)	Phl^+	0.345 ± 0.011
M114	Phl^-	<0.001
M114(pCU203)	Phl^+	0.373 ± 0.019

[a] Phl production was assayed in supernatants extracted from agar plates containing SA(+$FeCl_3$) medium with 7.5 g of agar per liter, previously overlaid with a confluent lawn of the respective *Pseudomonas* strain. Plates were incubated for 4 days at 14°C, prior to the collection of the supernatant (see Materials and Methods). Results are ± standard error.

concluded that F113G22 containing Tn*5*::*lacZY* maintained a stable Phl-negative phenotype in the sugar beet rhizosphere in soil.

Isolation of genes involved in Phl biosynthesis. DNA-DNA hybridization analysis revealed that F113G22 contained a single copy of Tn*5*::*lacZY* (data not shown). A genomic library of F113 total DNA was constructed in the broad-host-range vector pSUP106. Clones containing genetic determinants for inhibition towards *B. subtilis* were identified by complementation of the mutant F113G22. One transformant of F113G22 which exhibited pronounced inhibition towards *B. subtilis* was identified. The recombinant plasmid in this transformant (pCU203) contained a 6-kb genomic insert and in a F113G22 background produced 75% as much Phl as F113 on SA (+$FeCl_3$) agar (Table 1). The 6-kb genomic insert in pCU203 corresponded with the region of DNA in F113G22 in which the Tn*5*::*lacZY* was located. This was confirmed by Southern hybridization. F113 and F113G22 total genomic DNA was digested with *Eco*RI. Tn*5*::*lacZY* contains a single *Eco*RI restriction site; therefore, when the 6-kb genomic insert from pCU203 was used as a probe, F113 produced a single 10-kb band of hybridization whereas F113G22 produced two bands. The total molecular weight increase of these two bands corresponded to a fragment size increase of 12-kb equivalent to the size of Tn*5*::*lacZY* (data not shown).

Expression of gene(s) involved in Phl biosynthesis. pCU203 was transferred by conjugation into eight *Pseudomonas* strains, which were unable to produce but which were resistant to Phl. The presence of pCU203 in these *Pseudomonas* strains was confirmed by plasmid DNA isolation experiments. The plasmid DNA was subsequently restricted with appropriate restriction enzymes whose restriction sites in pCU203 were previously determined by mapping. The restriction profiles of all plasmid DNA preparations were compared. The profile of pCU203 isolated from F113G22 was similar when compared to those of pCU203 isolated from the eight *Pseudomonas* strains. Of this group, Phl production was detected (by HPLC analysis) only in strain M114, containing pCU203 (Table 1). Strain M114 containing the clone pCU203 produced 81% as much Phl in comparison to F113 on SA (+$FeCl_3$) agar. On the same medium, strain M114 (pCU203) exhibited increased inhibition towards *P. ultimum*, in comparison to strain M114 (Fig. 3). After gel electrophoresis, the banding patterns of *Eco*RI total genomic DNA digests of strains M114 and M114(pCU203) were similar (data not shown), confirming that M114(pCU203) was

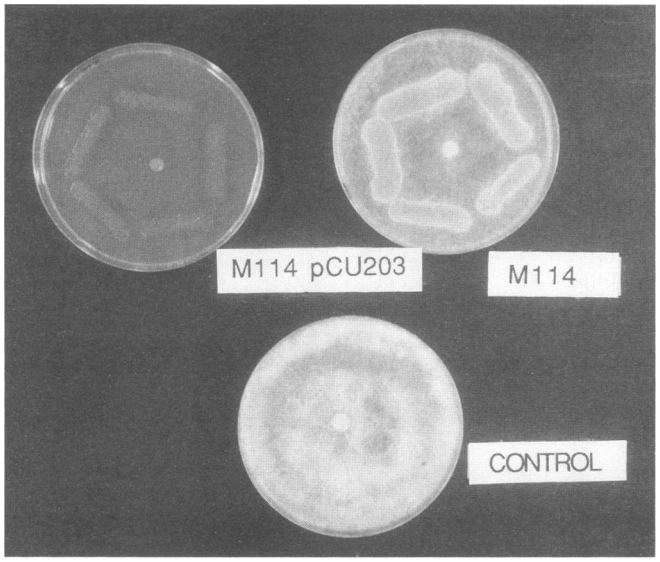

FIG. 3. Inhibition towards *P. ultimum* by *Pseudomonas* sp. strains M114 and M114 (pCU203) on SA (+$FeCl_3$) agar medium.

a derivative of M114. This result was also confirmed by randomly amplified primer DNA polymerase chain reaction (15a). Phl was not detected in the supernatant of the wild-type *Pseudomonas* sp. strains F63, R21/1, C21/1, OV6, C3, E1/7, and F2/2 or in their derivatives containing the recombinant plasmid pCU203.

Comparison of the biocontrol and root colonizing ability of strains M114 and M114(pCU203). The emergence of seeds inoculated with strain M114(pCU203) was significantly greater ($P < 0.05$) than that of seeds inoculated with M114 or untreated seeds and equivalent to that of seeds treated with the fungicides Thiram and Previcur N (Fig. 4). The rates of colonization of strains M114 and M114(pCU203) were not significantly different ($P > 0.05$) in the sugar beet rhizosphere, during the course of the experiment (Fig. 5). When rhizosphere soil from uninoculated roots was assayed on selective medium, no background level of indigenous *Pseudomonas* strains was obtained. The stability (± standard error) of pCU203 in M114 (assessed by the ability of colonies to exhibit chloramphenicol resistance) was 100%, 82% ± 11%, 89% ± 1%, and 89% ± 4% after 0, 5, 14, and 21 days, respectively. Ten colonies, isolated from the sugar beet rhizosphere at the final time of sampling (and resistant to chloramphenicol), produced the same amount of Phl as the M114(pCU203) isolate originally introduced into the soil (data not shown). Under laboratory conditions it was difficult to reproducibly obtain adequate fungal disease pressure in bioassays with field soil from test sites that were shown to have fungal infestation. However when significant differences were obtained between controls, i.e., commercially treated and untreated plants, M114(pCU203) produced a significant increase in germination.

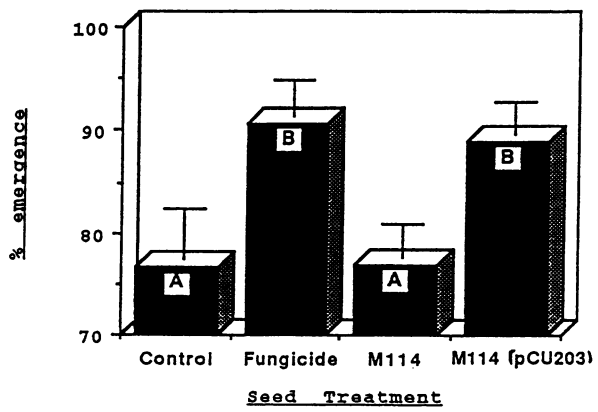

FIG. 4. Emergence of sugar beet seeds inoculated with *Pseudomonas* sp. strains M114 and M114(pCU203) and control (untreated) and fungicide-treated seeds in a soil subject to damping-off by *P. ultimum*. Columns containing the same letter were not significantly different at the $P < 0.05$ level by analysis of variance. Bars represent standard errors.

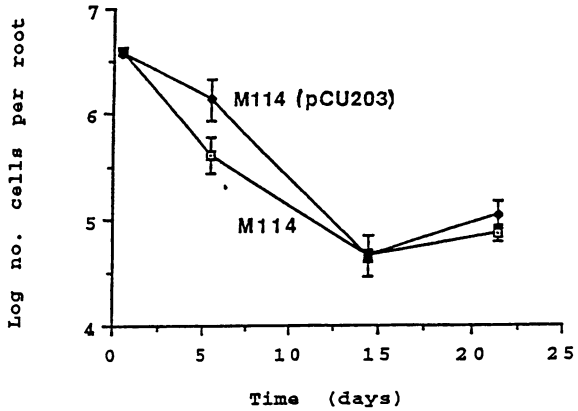

FIG. 5. Colonization of the sugar beet rhizosphere by *Pseudomonas* sp. strains M114 and M114(pCU203). Bars represent standard errors.

DISCUSSION

Previous studies showing that the production of Phl is involved in the suppression of soilborne plant fungal pathogens by *Pseudomonas* strains have been limited to gnotobiotic systems (12, 13). Thomashow et al. (21), reported that *P. fluorescens* 2-79, which produces the antibiotic phenazine-1-carboxylic acid, produced approximately 10-fold less antibiotic on wheat roots in unsteamed as compared to steamed soil and that production of phenazine-1-carboxylic acid was directly related to the suppression of *G. graminis* var. *tritici*. The data presented in this study suggest that the production of Phl by *Pseudomonas* strains is involved in the suppression of specific soilborne plant fungal pathogens in the unsterilized soil used in our system. It must be appreciated, however, that other factors such as colonization, competition, and bacterial numbers can also influence biocontrol especially when natural soils are employed.

To isolate genes involved in Phl biosynthesis, the transposon mutant F113G22, deficient in Phl production, was complemented with a plasmid library of strain F113. A plasmid, pCU203, was isolated which partially complemented F113G22 for Phl production. The reason(s) why clone pCU203 is unable to fully complement F113G22 is unclear. Haas et al. (9), in trying to complement a Phl-negative mutant of *P. fluorescens* CHA0, also reported isolating a cosmid which partially complemented strain CHA0 for Phl biosynthesis. From HPLC chromatograms, it seems likely that a cosmid (pMON5118) isolated by Vincent

et al. (22) failed to fully complement a Tn*5* mutant of *P. aureofaciens* for Phl production under the conditions reported.

Of the eight *Pseudomonas* strains into which the clone pCU203 was introduced Phl was found to be produced only by strain M114. It was confirmed that the restriction profile of pCU203 was not altered in any of the eight *Pseudomonas* strains, thus indicating that deletions of pCU203 had not occurred in any of the strains. In addition, the strains employed in this study were Phl resistant and consequently production of Phl would be unlikely to be deleterious to the organism. Therefore this would not appear to be a factor limiting the ability to detect Phl production in the strains.

Previous studies suggest that genes involved in the biosynthesis of antibiotics are often located in different genomic regions. For example, genes encoding the antibiotic oomycin A in *P. fluorescens* HV37A are located in at least three distinct genomic regions (8), while at least three loci on separate restriction fragments are involved in phenazine biosynthesis in *Pseudomonas* sp. strain 2-79 (4). It is therefore possible that the clone pCU203 contains only part of the total genomic region required for the biosynthesis of Phl and that M114, unlike the other *Pseudomonas* strains tested, possessed the necessary genomic region(s) required for complete biosynthesis. Alternatively, it may also be possible that pCU203 contains a gene(s) encoding a regulatory factor for Phl production. Such a gene(s) when introduced into a Phl-negative strain could be responsible for activating Phl production. Further experimental work will be necessary to understand the nature of the gene(s) involved.

The recombinant plasmid pCU203, in a M114 background, significantly enhanced the ability of M114 to act as a biocontrol agent against *P. ultimum* in soil. This result suggests that, by using pCU203, it may be possible to activate latent genes in other rhizobacteria with other desirable characteristics. In so doing, more efficient biocontrol agents may be produced.

The differences in the biocontrol ability of strain F113 compared with that of F113G22 and of strain M114(pCU203) compared with that of M114 suggest that Phl was produced in the sugar beet rhizosphere in soil. The Phl-negative derivative of strain F113 colonized seeds and roots of sugar beet as well as the three Tn*5*::*lacZY* derivatives of the parent strain. In addition, strain M114 colonized the seeds and roots of sugar beet as well as M114(pCU203). These results indicate that the production of Phl was not a major factor influencing the ability of these *Pseudomonas* strains to colonize sugar beet in soil. Previous reports have also shown that the production of other inhibitory compounds is not important in determining the ability of a bacterium to colonize the plant rhizosphere in soil. For example, Howie and Suslow (10) reported that a mutant of *P. fluorescens* Hv37a deficient in antibiotic biosynthesis exhibited reduced suppression towards *Pythium* spp. but colonized seeds and roots of cotton as well as the parent strain.

In conclusion, the results presented in this paper show that the cloning of genes involved in antibiotic biosynthesis offers the possibility to test the hypothesis that superior biocontrol agents can be designed by incorporating desirable traits into a single strain. Further analysis of the gene(s) involved in the pathway for Phl production will help determine the number and function(s) of the gene(s) involved and will be instrumental in transferring the ability to produce Phl into other bacterial strains.

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